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(57) Abstract

The present biotechnological approach for the production of polyhydroxyalkanoates (PHAs) uses microbial systems. The high production costs make them substantially more expensive than synthetic plastics. Engineering a novel pathway in eucaryotic cell systems is a beneficial alternative to the production of PHAs in bacteria. This pathway will initially produce free (C¿8) fatty acids from the fatty acid synthetic pathway through the action of thioesterase, that will then add a CoA moiety to the free fatty acid through the action of an acyl-CoA synthetase, that will produce 3--ketoacyl-CoAs from the acyl-CoA through the action of a thiolase, that will produce R-(-)-OH-acyl-CoAs from the 3-keto acid CoAs through the action of a dehydrogenase isoform from yeasts. These R-(-)-3-OH-acyl-CoAs will finally be used as substrate for the PHA synthase reaction.

(57) Abrégé

L'approche biotechnologique de la présente invention qui permet de produire des polyhydroxyalcanoates (PHA) utilise des systèmes microbiens. Les coûts de production élevés rendent ces derniers sensiblement plus coûteux que les plastiques synthétiques. La mise au point d'un nouveau mécanisme dans des systèmes cellulaires eucaryotes est une solution de remplacement intéressante à la production de PHA dans des bactéries. Ce mécanisme produit au départ des acides gras libres (C¿8) à partir du mécanisme de synthèse des acides gras par l'action de la thioestérase, ceci ajoutant ensuite une fraction CoA à l'acide gras sous l'effet d'une synthétase acyl-CoA, ce qui produit du 3--cétoacyl-CoAs à partir de l'acyl-CoA sous l'effet d'une thiolase, ce qui produit du R-(-)-OH-acyl-CoAs à partir du 3-cétoacide CoAs sous l'effet d'une isoforme déshydrogénase provenant de levures. Ces R-(-)-3-OH-acyl-CoAs sont ensuite utilisés en tant que substrats pour la réaction de synthétase de PHA.

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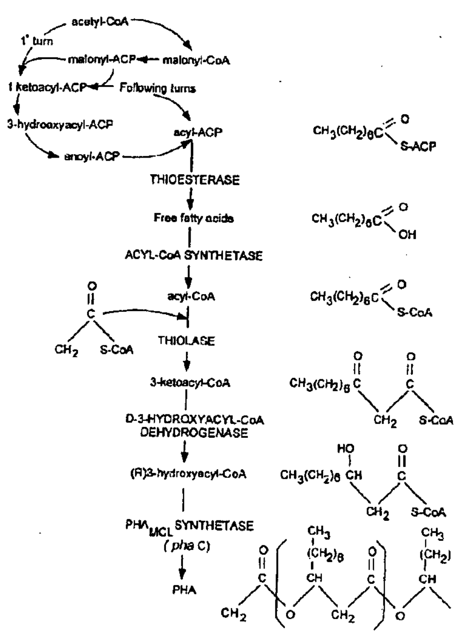
The present biotechnological approach for the production of polyhydroxyalkanoates (PHAs) uses microbial systems. The high production costs make them substantially more expensive than synthetic plastics. Engineering a novel pathway in eucaryotic cell systems is a beneficial alternative to the production of PHAs in bacteria. This pathway will initially produce free ($C_8$) fatty acids from the fatty acid synthetic pathway through the action of thioesterase, that will then add a CoA moiety to the free fatty acid through the action of an acyl–CoA synthetase, that will produce 3-ketoacyl–CoAs from the acyl–CoA through the action of a thiolase, that will produce R–(–)–OH–acyl–CoAs from the 3–keto acid CoAs through the action of a dehydrogenase isoform from yeasts. These R–(–)–3–OH–acyl–CoAs will finally be used as substrate for the PHA synthase reaction.



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Description

# METHOD FOR PRODUCING POLYHYDROXYALKANOATES IN RECOMBINANT ORGANISMS

The present invention relates to method for producing polyhydroxyalkanoates in recombinant organisms.

## BACKGROUND OF THE INVENTION

Plastic materials have become an integral part of contemporary life because they possess many desirable properties, including durability and resistance to degradation. Over the past 10-20 years, their widespread use have been increasingly regarded as a source of environmental and waste management problems. Industrial societies are now more aware of the impact of discarded plastic on the environment, and of their deleterious effect on wildlife and the aesthetic qualities of cities and forests. Problems associated with the disposal of waste and reduction in the availability of landfills have also focused attention on plastics, which accumulate in the environment at a rate of 25 million tonnes per year (Lee, 1996). These problems have created much interest in the development and production of biodegradable plastics. Biodegradable polymers are composed of material which can be degraded either by non-enzymatic hydrolysis or by the action of enzymes secreted by microorganisms. Estimates of the current global market for these biodegradable plastics range up to 1.3 billion kg per year (Lindsay, 1992).

Among the various biodegradable plastics available, there is a growing interest in the group of polyhydroxyalkanoates (PHAs). These are natural polymers produced by a variety of bacteria and they are 100% biodegradable. By changing the carbon source and bacterial strains used in the fermentation processes, PHA-biopolymers having a wide variety of mechanical properties have been produced. Their physical characteristics range from hard crystalline to elastic, depending on the composition of monomer units (Anderson & Dawes, 1990). The majority of PHAs are composed of R-(-)-3-hydroxyalkanoic acid monomers ranging from 3 to 14 carbons in length

(C3-C14). The simplest member of the family, P(3HB) (C4), is highly crystalline, relatively stiff, and becomes brittle over a period of days upon storage under ambient conditions (Barham *et al.*, 1984; De Koning *et al.*, 1992; Doi, 1990; Holmes, 1988). Therefore, attempts have been made to decrease the brittleness of P(3HB) either by incorporating comonomers such as P(3HV), by blending with other polymers or blending with chemically synthesized atactic P(3HB) (Holmes, 1988; Kumagai & Doi, 1992 a, 1992 b, 1992 c; Pearce & Marchessault, 1994).

The P(3HB-co-3HV) copolymer, developed by ZENECA under the tradename BIOPOL™, has improved mechanical properties compared to P(3HB). As the fraction of P(3HV) (C5) increases, the polymer becomes tougher, more flexible and have an higher elongation to break (Doi *et al.*, 1990). The medium-chain-length (MCL) PHAs are semicrystalline elastomers with a low melting point, low tensile strength, and high elongation to break. They thus have physico-chemical characteristics that make them more appealing than homogeneous P(3HB); they can even be used as a biodegradable rubber after cross linking by electron-beam irradiation (De Koning *et al.*, 1994; Gagnon *et al.*, 1992; Gross *et al.*, 1989; Preusting *et al.*, 1990).

PHAs have been shown to occur in over 90 genera of Gram-positive and Gram-negative bacteria species (Steinbüchel, 1991). Over 40 different PHAs have been characterized, with some polymers containing unsaturated bonds or various functional groups (Steinbüchel, 1991). Bacteria synthetise and accumulate PHAs as carbon and energy storage materials or as a sink for redundant reducing power under the condition of limiting nutrients in the presence of excess carbon sources (Byrom, 1994; Doi, 1990; Steinbüchel & Valentin, 1995). When the supply of the limiting nutrient is restored, the PHAs are degraded by intracellular depolymerases and subsequently metabolized as carbon and energy source (Byrom, 1990; Doi, 1990). The monomer 3HAs released from degradation of these microbial polyesters are all in the R-(-)-configuration due to the stereo specificity of biosynthetic enzymes (Anderson & Dawes, 1990). The molecular weights of polymers are in the range of $2X10^5$ to $3X10^6$ Daltons, depending on the microorganism and growth condition (Byrom, 1994).

PHAs accumulate in the cells as discrete granules, the number per cell and size of which can vary among the different species; 8 to 13 granules per cell of 0.2 to 0.5 $\mu$m diameter have been observed in *Alcaligenes eutrophus* (Byrom, 1994).

PHAs can be subdivided in two groups depending on the number of carbon atoms in the monomer units : short-chain-length-(SCL) PHAs, which contain 3-5 carbon atoms, and medium-chain-length-(MCL) PHAs, which contain 6-14 carbon atoms (Anderson & Dawes, 1990). This is mainly due to the substrate specificity of the PHA synthases that can only accept 3HA monomers of a certain range of carbon lengths (Anderson & Dawes, 1990). The PHA synthase of *Alcaligenes eutrophus* can polymerize C3-C5 monomers, but not C6 or higher. On the other hand, the PHA synthase of *Pseudomonas oleovorans* only accepts C6-C14 monomers. Of particular interest is the capacity of some PHA synthase to polymerize 3-hydroxy-, 4-hydroxy- and 5-hydroxy-alkanoates (Steinbüchel & Schlegel, 1991). Even though most of the PHA synthases examined to date are specific for the synthesis of either SCL- or MCL-PHAs, at least six cases were recently reported in which the bacteria were able to synthesize copolymer consisting of SCL and MCL units (Lee, 1996).

P(3HB) is the most widespread and thoroughly characterized PHA, and most of the knowledge has been obtained from *Alcaligenes eutrophus* (Steinbüchel, 1991). In this bacterium, P(3HB) is synthesized from acetyl-CoA by the sequential action of three enzymes (Figure 1). The first one, 3-ketothiolase, catalyses the reversible condensation of two acetyl-CoA moieties to form acetoacetyl-CoA. Acetoacetyl-CoA reductase subsequently reduces acetoacetyl-CoA to R-(-)-3-hydroxybutyryl-CoA, which is then polymerized by the action of PHA synthase to form P(3HB). A number of PHAs with different C3 to C5 monomers have been produced in *A. eutrophus*, the nature and proportion of these monomers being influenced by the type and relative quantity of the carbon sources supplied to the growth media (Steinbüchel & Valentin, 1995).

*Pseudomonas oleovorans* and most pseudomonades belonging to the ribosomal rRNA homology group I synthesize MCL-PHAs from various MCL-alkanes, alkanols, or alkanoates (Steinbüchel & Valentin, 1995). The composition of PHA produced is related to the substrate used for growth, with the polymer being mostly composed of monomers which are 2n carbons shorter than the substrates used. It was suggested that the acyl-CoA derived from alkanoic acids enter the ß-oxidation pathway and R-(-)-3hydroxyacyl-CoA intermediates used by the PHA synthase are generated either through reduction of 3-ketoacyl-CoA by a ketoacyl-CoA reductase, conversion of S-(+)-3hydroxyacyl-CoA normally produced by the pathway to the R-(-)- isomer by an epimerase, or the direct hydration of enoyl-CoA by an enoyl-CoA hydratase (Poirier *et al.*, 1995).

Most pseudomonades belonging to rRNA homology group I, except *P. oleovorans*, also synthesize MCL-PHAs when grown on substrates non related to fatty acids and alkanoates, such as gluconate, lactate, glycerol, and hexoses (Anderson & Dawes, 1990; Huijberts *et al.*, 1994; Timm & Steinbüchel, 1990). These substrates must be first converted into acetyl-CoA to be used for the PHAs biosynthesis. This suggests that, in theory, microorganisms, plants and even animals, must be able to synthesize PHA following the transfection of a limited number of genes. In these bacteria, three main pathways have been proposed for the synthesis of PHA precursors (Huijberts *et al.*, 1992, 1994).

(i) A detailed analysis of the composition of PHA produced by *P. putida* grown on glucose have shown that the monomers are structurally identical to the acyl-moieties of the 3-hydroxyacyl-ACP intermediates of *the novo* fatty acid biosynthesis. Since it has not been shown that PHA synthase can accept acyl-ACPs as substrates, these must therefore be converted to acyl-CoAs by a transacylase before entering the PHA pathway.

(ii) Fatty acid degradation by ß-oxidation is the main pathway when fatty acids are used as substrate.

(iii) It has been found that some of the monomeric units of PHA are one C2 unit longer than the fatty acid used as substrate. Chain elongation by condensation of an acetyl-CoA to the acyl-CoA has therefore been suggested.

A complex picture thus emerges in which the steps linking the different pathways implied and PHA synthesis are at present unknown (Figure 2). It is assumed, but not demonstrated, that the ultimate substrate for polymerization is the R form of the CoA-activated 3-hydroxy fatty acid intermediates. Expression of the synthases of *P. putida* in wild-type *E. coli* is not sufficient to produce PHA in this bacterium (Huisman, 1991). More genetic information from *Pseudomonas* spp. seems to be needed to enable PHA synthesis in *Escherichia coli*. We can speculate that the missing step in prokaryotic organisms other than pseudomonades is the formation of R-(-)-3-OH-acyl-CoA of more than 5C.

The bio(techno)logical approach for the production of PHAs use microbial systems. The major commercial drawback of the so-produced bacterial PHAs are their high production cost, making them substantially more expensive than synthetic plastics. At present, Zeneca produces approximately 1,000 tons per year of P(3HB-co-3HV) copolymer at a cost of approximately $16/kg. At a production rate of 10,000 tons per year or more, the most optimistic scenario would put the cost at $5/kg. With the cost of many synthetic plastics such as polypropylene and polyethylene, being less than $1/kg, PHA appear too costly for most low-value consumer products (Poirier *et al.*, 1995).

Engineering of novel pathways in eucaryotic cell systems seems to be a beneficial alternative to the production of PHAs in bacteria. On one hand, yeast and insect cells can be used as models to gain information on PHAs synthesis in eucaryotes (Hahn *et al.*, 1996; Sherman, 1996). On the other hand, a new possibility for the production of PHAs on a large scale and at costs comparable to synthetic plastics has arisen from the demonstration of their production in transgenic plants (Poirier *et al.*, 1992). Production of PHA on an agronomic scale could allow synthesis of biodegradable plastics in the

million ton scale compared to fermentation which produces material in the thousand ton scale (Poirier *et al.*, 1995). In addition, plant production of PHAs would use carbon dioxide, water and sunlight as raw materials to produce PHA in an environmentally friendly and sustainable manner.

Synthesis of PHB in plants was initially explored by expression of the PHB biosynthetic genes of A. eutrophus in the plant *Arabidopsis thaliana* (Poirier *et al.*, 1992). Although of no agricultural importance, this small oil seed plant was chosen for its extensive use as a model system for genetic and molecular studies in plants. These plants accumulated P(3HB) granules that were 0.2 to 0.5 $\mu$m in diameter in the nucleus, vacuole, and cytoplasm. However, the amount of P(3HB) accumulated was only 100 $\mu$g/g fresh weight. Furthermore, plants were impaired in their growth, probably due to the severe deviation of substrate from the mevalonate pathway which is essential for chlorophyll synthesis.

To avoid this problem and to improve polymer accumulation, further genetic manipulation have been carried out to divert reduced carbon away from endogenous metabolic pathways and to regulate the tissue specificity and timing of gene expression. The plastid was suggested to be the ideal location for P(3HB) accumulation because it is the location of high flux of carbon through acetyl-CoA. Genetically engineered genes of *A. eutrophus* were then successfully targeted to the plant plastids, where the enzymes were active (Nawrath *et al.*, 1995). The *A. eutrophus* PHA biosynthesis genes were modified for plastid targeting by fusing the transit peptide of ribulose biphosphate carboxylase to their N-terminal ends and were put under the control of the constitutive CaMV 35S promoter. The hybrid expressing the *A. eutrophus* PHA synthesis enzymes accumulated P(3HB) up to 10 mg/g fresh weight, representing ca. 14% of dry weight.

The knowledge acquired in this study is not only useful to optimise strategies for the production of PHB in recombinant organisms, but could also be used for the production of PHAs other than PHB, for example MCL-PHAs. For plant production of PHAs to become commercially viable, the genes must be transfected into a suitable

plant species which has the agronomic properties to provide high yields of PHA per hectare, at unlimited scale and at economic prices. Subcellular localization signals and promoters must be chosen which allow the enzymes utilized to intercept the desired plant metabolites for incorporation into the polymer.

Different strategies have been proposed for production of PHAs in plants. Substitution of cytoplasmic oil bodies by PHA granules, production of PHAs in glyoxysomes or production of PHAs in leucoplasts have been proposed to be carried out in lipid-accumulating tissues of oilseed crops, such as seed endosperm or fruit mesocarp (van der Leij & Witholt, 1995; Hahn *et al.*, 1996; Srienc & Leaf, 1996). In this tissue, triglycerides provide energy and carbon for germination of the new plant before establishment of photosynthesis. In contrast, PHAs would not be degraded in plants because of the absence of endogenous enzymes capable of hydrolyzing the polymer. Interfering with synthesis and degradation of fatty acids, in respectively plastids and glyoxysomes, by diverting energy into PHAs in this stage of development is likely to impair germination and/or seedling growth. As a post-harvest event this can be desirable. However, this inherent characteristic of the proposed strategy will create problems in the production of viable hybrid seeds. The expression of the enzymes during germination should be restricted to the second generation of seeds or fruit. For this, solutions will have to be found. It is probable that controlled expression of these genes will necessitate the use of promoters stimulated by external signals (Williams *et al.*, 1992).

Plastids are regarded as the most amenable targets for PHA production. The production in chloroplasts, directly coupled to *the novo* fatty acid synthesis has many advantages. First, every important crop can be used. Second, in leaves, fatty acid metabolism is not as important as in seeds and targeting to this tissue is not likely to impair growth of the plant. Third, it is the most direct way for PHAs production, since the plant does not have to produce long-chain fatty acids or triglycerol before diverting fatty acid degradation products into PHAs, like in the case of glyoxysomal degradative mechanisms. Fourth, as shown for the synthesis of PHB, compartmentalization in

plastids does not impair growth and then appear to be favoured over unrestricted synthesis in the cytosol.

*Pseudomonas aeruginosa* belongs to the group of pseudomonades of the rRNA homology group I that synthesize MCL-PHAs when grown either on alkanes or on unrelated substrates such as gluconate (Timm & Steinbüchel, 1990). A PHA synthase locus in *P. aeruginosa* was identified by the use of a $^{32}$P-labeled 30-mer synthetic oligonucleotide probe, whose sequence design was based on that of a highly conserved region of PHA synthases in *A. eutrophus* and *P. oleovorans* (Steinbüchel *et al.*, 1992). The organization of the locus consist of two genes coding for PHA synthases (*phaC1*, *phaC2*) separated by a gene coding for a putative PHA depolymerase (*phaD*), and a fourth gene (ORF3) downstream of *phaC2* with an unknown function (Timm & Steinbüchel, 1992). It has been shown that these synthases are similar to those found in *P. oleovorans*, who is unable to synthesize MCL-PHAs from unrelated substrates (Huijberts *et al.*, 1992).

As was shown in *P. aeruginosa*, intermediates of fatty acid biosynthesis and ß-oxidation are likely to contribute to the formation of PHA polymers. It is most likely that the intermediate precursors to PHA synthesis are either ketoacyl-CoA, S-(+)-3-OH-acyl-CoA, enoyl-CoA, or R-(-)-3-OH-acyl-ACP. However, since substrate specificity for PHA synthase has not yet been thoroughly tested, it is still unclear whether this enzyme could accept other derivative forms of 3-hydroxyacyl moieties, like for instance ACP derivatives. This can be of substantial impact on the choice of the best strategy for production in recombinant organisms: if the recombinant enzyme can accept, even at sub-optimum rates, ACP derivatives as substrate, then its targeting to chloroplasts would be the only required engineering alteration needed to induce PHA accumulation in leaf cells.

Monomeric units of PHAs, as it is the case for these of PHBs, are of the isomeric form R-(-)-; this has been repeatedly demonstrated by the analysis of hydrolysate from PHA granules. Enzymologic analysis also show that PHB synthases have a definite

specificity for R-(-)-3-OH-acyl-CoA as substrates. Although the substrate specificity of PHA synthases has not yet been thoroughly characterized with purified enzyme preparations, their high homology with PHB synthases and the analysis of their reaction product strongly suggest that they share a preference for R-(-)-3-OH-acyl CoA substrates with PHB synthases.

There are no demonstration of a metabolic pathway that would supply monomeric subunits to the polymerization reaction in Pseudomonades, nor in any other organisms. Known degradation pathways starting with acyl-CoAs produce S-(-)3-OH-acyl-CoAs and synthetic pathways produce R-(-)-acyl-ACPs, none of which can serve as substrate for the PHA synthesis reaction.

As further background, the following US Patent should be reviewed: 5,650,555; 5,502,273; 5,245,023; 5,610,041; 5,229,279; 5,534,432; 5,750,848; 5,663,063; 5,480,794; 5,750,848; 5,801,027; 5,298,421 and 5,250,430.

## SUMMARY OF THE INVENTION

This invention is directed at the production of polyhydroxyalkanoates in recombinant organisms, through the engineering of a new metabolic pathway which produces R-(-)-3-OH-acyl-CoAs monomeric subunits of adequate length to serve as substrates for the activity of PHA synthases.

More specifically, it describes the methodology that is used to produce transgenic organism with a new metabolic pathway that partially deviates fatty acids from their normal synthetic pathways, towards the formation of R-(-)-3-OH-acyl-CoAs that serves as substrates for the synthesis of hydroxyalkanoate polymers in chloroplasts.

The engineered synthetic metabolic pathway of the present invention initially produces free ($C_8$) fatty acids from the fatty acid synthesis pathway through the action of a thioesterase, that will then add a CoA moiety to the free fatty acid through the action of an acyl-CoA synthase, that will produce 3-(-)-ketoacyl-CoAs from the acyl-CoA through

the action of a thiolase, that will produce R-(-)-OH-acyl-CoAs from the 3-keto acid-CoAs through the action of a unique dehydrogenase isoform from yeast. These R-(-)-3-OH-acyl-CoAs will finally be used as substrate for the PHA synthase reaction.

Thus according to the present invention there is provided a method for the production of polyhydroxyalkanoates comprising: selecting a transgenic organism comprising a foreign DNA sequence encoding an enzyme having dehydrogenase activity, which will produce a R-(-)-hydroxyacyl-CoA from a keto acid-CoA, wherein said R-(-)-hydroxyacyl-CoA will serve as a substrate for polyhydroxyalkanoate synthase; and producing said polyhydroxyalkanoate.

Further, according to the present invention there is provided a method for producing a polyhydroxyalkanoate in a host comprising: selecting a host for expression of genes encoding enzymes required for synthesis of a polyhydroxyalkanoate; introducing into said host structural genes encoding enzymes selected from the group consisting of: a thioesterase, an acyl-CoA synthetase, a thiolase, a hydroxyacyl-CoA dehydrogenase, and a polyhydroxyalkanoate synthase; expressing the enzymes encoded by the genes; and providing the appropriate substrates for the expressed enzymes to synthesis the polyhydroxyalkanoate.

According to the present invention there is also provided a cloning vector comprising foreign DNA encoding an enzyme having dehydrogenase activity, which will produce R-(-)-hydroxyacyl-CoA from a keto acid-CoA. According to one embodiment of the invention the cloning vector further comprises a DNA sequence encoding an enzyme having thioesterase activity; an enzyme having acyl-CoA synthetase activity; an enzyme having thiolase activity; and an enzyme having polyhydroxyalkanoate synthase activity.

According to the present invention there is also provided a host cell comprising foreign DNA encoding an enzyme having dehydrogenase activity, which will produce R-(-)-hydroxyacyl-CoA from a keto acid-CoA. According to one embodiment of the

invention the host cell further comprises a DNA sequence encoding an enzyme having thioesterase activity; an enzyme having acyl-CoA synthetase activity; an enzyme having thiolase activity; and an enzyme having polyhydroxyalkanoate synthase activity.

According to the present invention there is also provided a transgenic organism comprising foreign DNA encoding an enzyme having dehydrogenase activity, which will produce R-(-)-hydroxyacyl-CoA from a keto acid-CoA. According to one embodiment of the invention the transgenic organism further comprises a DNA sequence encoding an enzyme having thioesterase activity; an enzyme having acyl-CoA synthetase activity; an enzyme having thiolase activity; and an enzyme having polyhydroxyalkanoate synthase activity. In one example of this embodiment the transgenic organism is a plant.

## BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

**FIGURE 1** shows the pathway for the production of P(3HB) by *Alcaligenes eutrophus*.

**FIGURE 2** is a hypothetical pathway for the synthesis of PHAs from Pseudomonades.

**FIGURE 3** is the synthetic pathway of the present invention showing the five steps involved in the production of medium chain length PHAs.

**FIGURE 4** shows the DNA constructs of the present invention.

**FIGURE 5** shows the DNA sequences for pKitmus/Rbsck-DH-3' nc, Figure 5a (SEQ ID NO: 1) (The sites *SalI*, *SphI*, *ApaI*, *Eco*RI and *SmaI* are underlined and the ATG site is in bold); pUC/35S.C4PPDK.DH.3' nc, Figure 5b (SEQ ID NO: 2) (*XhoI*, *SphI*, *ApaI* and *Eco*RI sites are underlined and the ATG of the protein is in bold); and pCambia/RbscK-DH-3' nc, Figure 5c (SEQ ID NO: 3) (*SalI*, *SphI*, *ApaI*, *Eco*RI and *SmaI* sites are underlined and the ATG of the protein is in bold.

**FIGURE 6** shows the dehydrogenase activity in E.coli. The monitoring activity is shown in Figure 6a and the linear part of the graph that represents the D-3-hydrozyacyl-CoA dehydrogenase activity is shown in Figure 6b.

**DESCRIPTION OF PREFERRED EMBODIMENT**

The present invention is directed at the production of polyhydroxyalkanoates in recombinant organisms, through the engineering of a new metabolic pathway which produces R-(-)-3-OH-acyl-CoAs monomeric subunits of adequate length to serve as substrates for the activity of PHA synthases.

More specifically, the present invention is directed to a methodology that is used to produce transgenic organisms with a new metabolic pathway that partially deviates fatty acids from their normal synthetic pathways, towards the formation of R-(-)-3-OH-acyl-CoAs that serves as substrates for the synthesis of hydroxyalkanoate polymers in chloroplasts. The new synthetic pathway of the present invention is depicted in Figure 3.

In one embodiment of the present invention the transgenic organism is a plant or any organ of a plant where there is active plastid activity.

According to the present invention examples of suitable plants include but are not limited to Arabidopsis, tobacco, alfalfa and tuber plants such as potato, sweet potato, beet and cassava.

Prior to the present invention, there was no demonstration of a metabolic pathway that would supply monomeric subunits to the polymerization reaction in Pseudomonades, nor in any other organisms. Known degradation pathways starting with acyl-CoAs produce S-(-)3-OH-acyl-CoAs and synthetic pathways produce R-(-)-acyl-ACPs, none of which can serve as substrate for the PHA synthesis reaction. Thus, the present invention is directed to a synthetic pathway that will produce R-(-)-OH-acyl-CoAs from 3-keto acid-

CoAs through the action of a dehydrogenase isoform from yeast. These R-(-)-OH-acyl-CoAs will then serve as a substrate for the PHA synthase reaction.

According to the present invention the term polyhydroxyalkanoate, is intended to include a polymer of R-(-)-3-hydoxyalkanoic acid monomers from about 3 to about 14 carbons in length. In one embodiment of the present invention the PHA synthase from *Pseudamonas aeruginosa* is used in the last step of the synthetic pathway. This enzyme prefers R-(-)-3-OH-acyl-CoAs of C6 to C14 as a substrate.

The biosynthetic pathway of the present invention involves five enzymes: a thioesterase, a acyl-CoA synthetase, a thiolase, a D-3-hydoxyacyl-CoA dehydrogenase and a PHA synthase.

The first reaction is catalyzed by a thioesterase. In one embodiment of the present invention the enzyme has been cloned from a cDNA library from *Cuphea hookeriana*, a Mexican bush plant which accumulates up to 75% of C8:0- and C10:0-fatty acids in seeds. This clone *Cl FatB2* (GenBank accession # U39834) has been expressed in *E. coli* where it exhibited a high specificity for C8:0 and C10:0-ACPs as substrates. In the chloroplasts, this enzyme removes C8- and C10-acyl-ACPs from the fatty acid synthetic pathway and releases free medium-chain length fatty acids in the stroma much as endogenous thioesterases do with C16- and C18-acyls-ACPs in the fatty acid synthetic pathway (Dehesh, K. et al., 1996, The Plant Journal 9(2): 167-172).

The second reaction is catalysed by an acyl-CoA synthetase. In one embodiment of the invention, the enzyme was isolated from *Pseudomonas oleovorans*, a bacteria which accumulates $PHAs_{mcl}$ (medium-chain length polyhydroxy alkanoates). The enzyme is encoded by gene K of operon alkBFGHJKL which is responsible for alkanoate synthesis. This acyl-CoA synthetase (GenBank accession # X65936) is specific to medium-chain length fatty acids (van Beilen, J.B. et al. (1992) DNA sequence determination and functional characterization of the OCT-plasmid-encoded alkJKL genes of *Pseudomonas oleovorans*. Molecular Microbiology 6(21): 3121-3136).

The third reaction is catalysed by a keto thiolase. This reaction is a condensation reaction which will add one acetyl-CoA moiety to the acyl-CoA, thus releasing one CoA molecule. This condensation reaction is reversible, and creates a 3-keto acyl CoA with two extra carbon. The products following this reaction will be therefore C10- and C12-3-OH-acyl CoAs and free CoA. The enzyme described in one example of the present invention has been isolated from *Brassica napus*, in which it is part of the oxidation pathway (Olesen, C. J. et al. (1997) The glyoxysomal 3-ketoacyl-CoA thiolase precursor from *Brassica napus* has enzymatic activity when synthesized in *E. coli* (FEBS Letters 6(21): 138-140; GenBank accession # X93015).

The fourth reaction, according to the present invention, is catalysed by a yeast 3-hydroxyacyl-CoA dehydrogenase which produces R-(-)-3-OH-acyl-CoAs (Hiltunen, J.K. et al. (1992) Peroxisomal multi functional B-oxidation protein of *Saccharomyces cerevisiae*. J. Biol. Chem. 267: 6646-6653; GenBank accession # M86456). Homologs of this 3-keto-acyl-CoA dehydrogenases usually produce S-(-)-3-OH-acyl-CoAs in the B-oxidation pathway. The 3-hydroxyacyl-CoA dehydrogenase domain of the multi functional protein (MFP) of yeasts exhibits this unique catalytic property. Thus the product of this reaction will be R-(-)-3-OH-decanoyl-CoA and R-(-)-3-OH-dodecanoyl-CoA. Both molecules can serve as substrate for the polymerization reaction catalyzed by PHA synthases.

The last reaction of this embodiment is catalysed by a PHA synthase from *Pseudomonas aeruginosa*, which accumulates large amounts of PHA granules in nutrient stress conditions (Timm, A. and Steinbüchel, A., 1992) Cloning and molecular analysis of the poly(3-hydroxyalkanoic acid) gene locus of *Pseudomonas aeruginosa* PAO1. Eur. J. Appl. Microbiol. 209: 15-30; GenBank accession # X66592). Analysis of depolymerization products shows that this enzyme uses R-(-)-3-OH-acyl-CoAs of C6 to C14 in length as substrates, with an apparent preference for C10 and C12 R-(-)-3-OH-acyl-CoAs.

In one embodiment of the present invention, each of these genes is sub-cloned in an appropriate expression vector. In one embodiment of this invention the host is a plant cell and any know plant expression vector can be used according to the present invention. Said plant expression vector can contain a promoter sequence, a 5'UTR sequence, a chloroplast transit peptide sequence, the complete coding sequence of the gene, a stop codon, an a 3'UTR region containing a eukaryotic polyadenylation signal and a polyadenylation site. The polyadenylation signal is usually characterized by effecting the addition of polyadenylic acid tracks to the 3' end of the mRNA precursor. Polyadenylation signals are commonly recognized by the presence of homology to the canonical form 5' AATAAA-3' although variations are not uncommon.

Examples of suitable 3' regions are the 3' transcribed non-translated regions containing a polyadenylation signal of *Agrobacterium* tumor inducing (Ti) plasmid genes, such as the nopaline synthase (*Nos* gene) and plant genes such as the soybean storage protein genes and the small subunit of the ribulose-1, 5-bisphosphate carboxylase (ssRUBISCO) gene. The 3' untranslated region from the structural gene of the present construct can therefore be used to construct chimeric genes for expression in plants.

The chimeric gene construct of the present invention can also include further enhancers, either translation or transcription enhancers, as may be required. These enhancer regions are well known to persons skilled in the art, and can include the ATG initiation codon and adjacent sequences. The initiation codon must be in phase with the reading frame of the coding sequence to ensure translation of the entire sequence. The translation control signals and initiation codons can be from a variety of origins, both natural and synthetic. Translational initiation regions may be provided from the source of the transcriptional initiation region, or from the structural gene. The sequence can also be derived from the promoter selected to express the gene, and can be specifically modified so as to increase translation of the mRNA.

To aid in identification of transformed plant cells, the constructs of this invention may be further manipulated to include plant selectable markers. Useful selectable markers

include enzymes which provide for resistance to an antibiotic such as gentamycin, hygromycin, kanamycin, and the like. Similarly, enzymes providing for production of a compound identifiable by colour change such as *GUS* (β-glucuronidase), or luminescence, such as luciferase are useful.

This invention is directed at any means by which the genes of interest can be transfected in a plant providing it results in stable integration and expression. Preferred means are, *Agrobacterium* mediated DNA transfer which requires T-DNA borders, and selectable markers; DNA bombardment, which requires selectable markers, and electroporation which can in some cases be used without screenable markers. These various cloning and plant transformation methods are well know in the art. For reviews of such techniques see for example Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academy Press, New York VIII, pp. 421-463 (1988); and Geierson and Corey, *Plant Molecular Biology*, 2d Ed. (1988).

Also considered part of this invention are transgenic organisms containing the chimeric gene construct of the present invention. In one embodiment of this invention the transgenic organism is a plant. Methods of regenerating whole plants from plant cells are known in the art, and the method of obtaining transformed and regenerated plants is not critical to this invention. In general, transformed plant cells are cultured in an appropriate medium, which may contain selective agents such as antibiotics, where selectable markers are used to facilitate identification of transformed plant cells. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be used to establish repetitive generations, either from seeds or using vegetative propagation techniques.

When specific sequences are referred to in the present invention, it is understood that these sequences include within their scope sequences that are "substantially similar" to said specific sequences. Sequences are "substantially similar" when at least about 80%, preferably at least about 90% and most preferably at least about 95% of the

nucleotides match over a defined length of the molecule. Sequences that are "substantially similar" include any substitution, deletion, or addition within the sequence. DNA sequences that are substantially similar can be identified in Southern hybridization experiments, for example under stringent hybridization conditions (see Maniatis et al., in Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory (1982) p 387 to 389).

The specific sequences, referred to in the present invention, also include sequences which are "functionally equivalent" to said specific sequences. In the present invention functionally equivalent sequences refer to sequences which although not identical to the specific sequences provide the same or substantially the same function. DNA sequences that are functionally equivalent include any substitution, deletion or addition within the sequence.

Since the creation of the novel metabolic pathway requires the simultaneous expression of 5 different transgenes, 5 independent transformants can be produced, and genotypes containing the 5 transgenes are produced by repeated crossing of mono-transgenics and selection. Alternately, series of genes can be co-transfected on single constructs, thus reducing the need for extensive crossing. Other means of integrating the novel genes in chloroplasts also include the direct transfection of DNA on chloroplastic DNA by recombination using homologous or heterologous border sequences. Using this methodology, polycistronic constructs (multiple gene construct under the control of a single promoter) could be used to bring the 5 modifications. These methods are well known to persons of skill in the art.

While this invention is described in detail with particular reference to preferred embodiments thereof, said embodiments are offered to illustrate but not limit the invention, as shown in the following example.

**EXAMPLES**

**PHA synthase**

Cloning of the PHA synthase gene PhaC1 (X66592,Timm, 1992) was performed by PCR amplification of genomic DNA from *Pseudomonas aeruginosa* strain PAO1 with the "Expand" system from Boehringer Mannheim. Template DNA was extracted and purified. The primer at the 5' terminus was 5'GATC GCATGC GAAGGATTTC TATGAGTCAG3'; it contains the SphI and XmnI restriction sites upstream from the ATG. The primer at the 3'terminus was 5'GATC GAATTC TCATCGTTCATGCACGTAGG3'; a EcoR1 site had been introduced downstream of the stop codon. Conditions for the PCR were:

94°C – 2'

| | |
|---|---|
| denaturation : 94°C –10'' | 10 cycles |
| annealing : 55°C – 30'' | |
| polymerization : 68°C – 4' | |

| | |
|---|---|
| denaturation : 94°C –10'' | 20cycles |
| annealing : 55°C – 30'' | |
| polymerization : 68°C – 4' d 20''/cycle | |

68°C – 7'

The PCR products formed were separated on agarose gels and the band corresponding to the expected size was removed from the gel. Purified DNA was then digested with SphI/EcoRI, and the 1692 pb fragments were cloned in pUC18. Complete homology of the selected clone was verified by sequencing. The synthase gene was then cloned at the SphI/EcoRI sites of pGEM-7Zf (Promega) containing the 3'non coding region (3'nc) of SSU-rubisco gene RbcsK (Khoudi et al 1997) previously cloned at the EcoRI/SmaI site. The SphI/SacI fragment (2138 pb)

containing PhaC1 + 3'nc was then sub-cloned into Litmus28 (NE Biolabs) containing the complete 5' region of RbcsK with the promoter and transit peptide (5' RbcsK), previously cloned at the SalI/SphI site. Finally, a fragment of 4112 bp containing the 5'RbcsK + PhaC1 + 3'nc was cloned into the SalI/SacI site of pBI 101.2 (Clontech), removing then the GUS gene. This construct was then transfected to *Agrobacterium tumefaciens* strain LBA4404, and incorporated in the genome of selected plant cells cells through co-cultivation with transgenic *A. tumefaciens*, as described by Desgagnés et al (1995) Plants were regenerated from transgenic cells, and leaf tissue is used for the selection of the best transgenic lines by Northern analysis. PHA synthase activity of the selected lines was then measured in clarified leaf extracts as described in the prior art.

**Hydroxyacyl-CoA dehydrogenase**

The domain of the multi functional b-oxidation protein (MFP) (M86456) (Hiltunen, 1992) which encodes for R-3-hydroxyacyl-CoA dehydrogenase was amplified by PCR from *Saccharomyces cerevisiae*. It has been shown that the protein contains two activities: a 2-enoyl-CoA hydratase 2, converting trans-2-enoyl-CoA to R-3-hydroxyacyl-CoA, and a R-3-hydroxyacyl-CoA dehydrogenase, converting R-3-hydroxyacyl-CoA to 3-ketoacyl-CoA. A truncated version of MFP lacking 271 carboxyl-terminal amino acids was also overexpressed and purified and it was shown that it has only the R-3-hydroxyacyl-CoA dehydrogenase activity. These results clearly demonstrate that the b-oxidation of fatty acids in the yeast follows a previously unknown stereochemical course, namely it occurs via a R-3-hydroxyacyl- CoA intermediates.

The expression of the truncated version in chloroplasts of plants with a medium-chain length- specific thioesterase, an acyl-CoA synthetase and a thiolase will allow the production of R-3-hydrozyacyl-CoAs, the substrate of the PHA synthase.

Template genomic DNA was extracted and purified from S. cerevisiae as described in Current Protocols in Molecular Biology, 1997, section 13.11.1-13.11.4. The 5' primer

used contains the SphI site upstream of the ATG (GATCGCATGCTAATGCCTGGAAATTTATCCTTC) and the 3'primer has an ApaI site downstream a newly introduced stop codon (GATCGGGCCC TTACGGGTTGATAGTGTTGCGACT). The PCR conditions are described above, except that the annealing temperature used was 50°C. The PCR fragment was purified, digested *SphI/ApaI* and the 1799 bp fragment was cloned in pKitmus/RbscK-3'nc. This vector is a pLitmus28 derivative that contains a cassette for chloroplasts expression. This cassette has in 5' the promoter of the small subunit of the ribulose 1,5-biphosphate carboxylase (rubisco) of alfalfa, its 5' non-translated region and the targeting signal to chloroplasts, followed by a multiple cloning site (MCS), and in 3', the 3' non coding region of rubisco (Khoudi, et al.,1997) . The region in 5' is a 1978 bp *SalI/SphI* fragment that has at its 3' end the sequence that codes for the 58 amino acids of the transit peptide, followed by the ATG of the mature protein. This ATG is found to be the one in the *SphI* site. The dehydrogenase clone in the MCS is then in frame with the targeting signal, having a leucine in between the ATG of the mature rubisco protein and the one of the gene. The 3' non coding region is a 441 bp EcoRI/SmaI fragment that has the two PolyA signals of the small subunit of rubisco.

A potential clone has been sequenced with these primers:

| | | |
|---|---|---|
| Jonc. Rub. | 5' | AAGTCCATGGCTGGCTTCCCA |
| Rub 1023r | 5' | AGATAGTAAATTCTCAAATGAATTC |
| DHSc125c | 5' | TTGACAGGTGGCTATAAG |
| DHSc498r | 5' | CGTTTCTGCATGAGGAGC |

And a wild type clone, pKitmus/RbscK-DH-3'nc #9, has been conserved for further manipulations (Figure 5a).

The gene was put in a cassette for transient expression. Plasmid 35SC4PPDK-sGFP-TYG-nos was obtained from Jen Sheen at the Department of Molecular Biology at Massachusetts General Hospital and contains the following: the 35S-C4PPDK promoter flanked by *XhoI* and *BamHI* sites; a gene encoding GFP flanked by BamHI and *PstI* sites that contains an amino acid change at position 65 for increased

fluorescence and whose codon usage is optimized for plant expression; and a polyadenylation sequence flanked by *Pst*I and *Eco*RI sites. The *Bam*HI site after the promoter was changed for a *Sph*I site by a treatment of the vector digested *Bam*HI with the Klenow and its ligation with a *Sph*I linker. The vector produced was then digested *Eco*RI, treated with the Klenow and digested *Sph*I to yield a *Sph*I/blunt vector fragment of 3290 bp that has lost the gene GFP and the NOS. pKitmus/RbscK-DH-3'nc was digested SphI-SmaI and the 2198 bp fragment containing the dehydrogenase and the 3'non coding region of alfalfa rubisco was ligated in the vector to produce pUC/35S.C4PPDK.DH.3'nc, a 5488 bp clone used in the transient expression experiment (Figure 5b).

For plant expression, the cassette containing the alfalfa rubisco promoter, the dehydrogenase and the 3' non coding region of alfalfa rubisco was obtained by a *SalI*/*Sma*I digestion of pKitmus/RbscK-DH-3'nc #9 and cloned in pCambia 2300. The clone formed is called pCambia/ RbscK-DH-3'nc (Figure 5c).

Diagrams of the constructs are shown in Figure 4, and the sequences files are found in Figure 5.

For the enzymatic activity of the (D)-3-hydroxyacyl-CoA dehydrogenase in *E. coli*, a 2XYT.Ap liquid culture (5 mL) is innoculated with a single colony of DH5$\alpha$ : pTRCN/FOX2 and the culture is placed under agitation at 30°C for 16 hours. This overnight culture is used to innoculate (1 %) 50 mL of 2XYT.Ap media. The cultures are put at 30°C under agitation until the $OD_{600nm}$ reach 0.6. IPTG (0.4 mM) is added and the culture is incubated for another 4 hours. The cells are collected by centrifugation (15 min./5 000 g/4°C) and stored at -80°C. The cells are resuspended in 20 mM $KH_2PO_4$ buffer (pH 7.0), 0.5 mM DTT, 0.1 mM PMSF and are disrupted by sonication with pulses of 0.2 sec for a total period of 20 sec. The cells are returned to ice for cooling purposes and the sonication procedure is repeated two more times to ensure lysis. The extract is clarified by centrifugation in a microfuge (12 000g/15 min./4°C) prior to activity measurements.

The dehydrogenase reaction measured the oxidation of a 3-hydroxyacyl-CoA in a 3-ketoacyl-CoA and is followed by monitoring the formation of the $Mg^{2+}$ complex of 3-ketoacyl-CoA at 303 nm. The incubation mixture consisted of 50 µmol Tris-Cl (pH 9.0), 50 µg bovine serum albumin, 50 µmol KCl, 1 µmol $NAD^+$, 25 µmol $MgCl_2$, 1 µmol pyruvate and 10 µg lactate dehydrogenase in a total volume of 1 mL. The lactate dehydrogenase allows the regeneration of $NAD^+$ using the pyruvate as substrate. The reaction is monitored at room temperature with all the components of the incubation mixture and then 10 µg of L-3-hydroxyacyl-CoA dehydrogenase is added. After about 1 minute, 50 nmol of the substrate DL-3-hydroxyoctanoyl-CoA is added. When the $OD_{303nm}$ is stabilized, meaning that the L-3-hydroxyoctanoyl-CoA is completely oxidized, then the extract (1%) is added. The reaction is monitored for another 5 minutes.

The R-3-hydroxyacyl-CoA dehydrogenase activity was measured in DH5$\alpha$ overexpression the MFP Fox2 gene of *Saccharomyces cerevisiae*. The extinction coefficient used for the 3-ketooctanoyl-CoA is 14.5 X $10^3$ $cm^{-1}$ $M^{-1}$. The monitoring activity is shown on Figure 6a and the linear part of the graph that represents the R-3-hydroxyacyl-CoA dehydrogenase activity is shown in Figure 6b. The specific activity is 0.81 Units/mg protein.

Transient expression of the 3-hydroxyacyl-CoA dehydrogenase in plant cells was done by transformation of *Arabidopsis thaliana* protoplasts with pUC/35S.C4PPDK.DH.3'nc (Sheen, J., et al., 1995). For the enzymatic activity of the (D)-3-hydroxyacyl-CoA dehydrogenase in plant cells, *Arabidopsis thaliana* protoplasts are harvested by centrifugation (115 g) and the supernatant is removed. An aliquot (14 µL) of 7X stock of protease inhibitor stock is added to the sample and the sample is brought to a final volume of 100 µL with a solution containing 20 mM $KH_2PO_4$ buffer (pH 7.0), 0.5 mM DTT, 0.1 mM PMSF. The 7X stock of protease inhibitors is prepared by dissolving one "Complete Mini Protease Inhibitor Tablet"

(Boehringer Mannheim) in 1.5 mL 20 mM $KH_2PO_4$ buffer (pH 7.0), 0.5 mM DTT, 0.1 mM PMSF. The protoplasts are disrupted in a 1.5 mL centrifuge tube using a pellet pestle mixer (Kontes) for 30 seconds. Soluble proteins are separated from insoluble proteins by centrifugation at maximum speed in a microcentrifuge (10 min, 4°C). Dehydrogenase activity was measured as described above. The R-3-hydroxyacyl-CoA dehydrogenase activity was measured in protoplasts of *Arabidopsis thaliana* transformed with pUC/35S.C4PPDK.DH.3'nc. The extinction coefficient used for the 3-ketooctanoyl-CoA is 14.5 X $10^3$ $cm^{-1}$ $M^{-1}$.

*Arabidopsis thaliana* plants were transformed with pCambia/RbscK-DH-3'nc following a floral dip protocol (Clough, S.J., et al. 1998) and using the *Agrobacterium tumefasciens* strain GV3101/pMP90 (Koncz, C., et al., 1986). For the enzymatic activity of the (D)-3-hydroxyacyl-CoA dehydrogenase in plants, leaves of *Arabidopsis thaliana* expressing the enzyme are collected and grinded in 5 volumes of extraction buffer containing 50 mM Tris-Cl buffer (pH 8.0), 0.4% β-mercaptoethanol, 2 mM PMSF. The extract is clarified on Miracloth and centrifuged (12 000g/15 min./4°C). The supernatant is desalted on a Sephadex G-25 PD-10 column (Pharmacia) eluting in a buffer containing 20 mM $KH_2PO_4$ buffer (pH 7.0), 0.5 mM DTT, 0.1 mM PMSF. Dehydrogenase activity was determined as described above. The R-3-hydroxyacyl-CoA dehydrogenase activity was measured in plants of *Arabidopsis thaliana* transformed with pCambia/RbscK-DH-3'nc. The extinction coefficient used for the 3-ketooctanoyl-CoA is 14.5 X $10^3$ $cm^{-1}$ $M^{-1}$.

**Keto-acyl CoA thiolase**

Cloning of the thiolase gene (X93015) (Olesen, 1997) was performed by PCR amplification of genomic DNA from *Brassica napus* as described above, except that the annealing temperature used was 50°C. Template DNA was prepared from B. napus leaves as described by Rogers & Bendich, Plant Mol. Biol., 1988, A6:1-10. The primer at the 5' end was 5'-GATC<u>GCATGC</u>TAGCTGGGGACAGTGCTGCGTATC-3' with an added SphI site, and at the 3' end 5'-GATC<u>GAATTCCTA</u>ACGAGCGTCCTTGGACAAAAG-3'

with an EcoRI site downstream of the stop codon. Primers were selected so that amplification would be initiated at position 106 of the cDNA and therefore eliminate the N-terminal targeting signal for glyoxysomes. The gel-purified PCR products were digested with SphI/EcoRI and cloned in a derivative of pLitmus 28 modified as described above for cloning of yeast dehydrogenase. Suitable clones are fully sequenced. Sequence was compared with the published cDNA (1389 pb), although amplicons were produced from genomic DNA template. Homologous amplicons with introns and without the targeting signal are 2568-bp in size. The whole construct with the homologous gene was sub-cloned at the SalI/SmaI site in pBI 101.2. Transformation of A. tumefaciens, transformation of selected plants, and regeneration of transgenic lines was performed as described above. Selection of best transgenic lines was performed with Northern hybridization.

**Acyl-ACP thioesterase**

A gene encoding for a thioesterase with specificity for 8:0 and 10:0-ACP substrates (U39834) (Dehesh, 1996) was amplified by PCR from *Cuphea hookeriana* as described above, except that the annealing temperature used was 60°C. Template genomic DNA was prepared with the Qiagen Genomic Tip Protocol as described by the manufacturer. The primer at the 5'end was 5'-GATCTCTAGA ATGGTGGCTGCTGCAGCAAGTTCCG-3' with a XbaI site upstream of the ATG; the primer at the 3' end was 5'-GATCGGGCCC CTAAGAGACCGAGTTTCCATTTGAAG-3' with an ApaI site downstream of the stop codon. The PCR product was cloned at the XbaI/ApaI site in the plant vector pCambia 2300, modified to harbor the RbscK promotor (SalI/SphI) with the transit peptide, a multiple cloning site between SphI and EcoRI and the 3' non coding region of RbscK (EcoRI/SmaI). Transformation of *A. tumefaciens*, transformation of selected plants, and regeneration of transgenic lines was performed as described above. Selection of best transgenic lines was performed with Northern hybridization.

**Acyl-CoA Synthetase**

Cloning of the acyl-CoA synthetase gene (X65936) (van Beilen, 1992) was performed by PCR amplification of genomic DNA from *Pseudomonas oleovorans*. Template DNA was prepared as described in Current Protocols in Molecular Biology, 1997, 2.4.1-2.4.2. The primer at the 5'-end was GATCGGATCC ATGTTAGGTCAGATGATGCGT-3' with a BamHI site upstream of the ATG; the primer at the 3' end was 5'-GATCGAATTC TTATTCACAGACAGAAGAACT-3' with an EcoRI site downstream of the stop codon. The PCR product was cloned in the BamHI/EcoRI site of pLitmus 28 modified as described above for cloning of yeast dehydrogenase. Suitable clones were fully sequenced. Wjole constructs were then sub-cloned into pBI.101.2. Transformation of *A. tumefaciens*, transformation of alfalfa and tobacco plants, and regeneration of transgenic lines was performed as described above. Selection of best transgenic lines was performed with Northern hybridization.

All scientific references and patent documents are incorporated herein by reference.

The invention as herein described can be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the present invention. All modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

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# Claims

**THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OF PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:**

1. A method for producing a polyhydroxyalkanoate comprising:
selecting a transgenic organism comprising a foreign DNA sequence encoding an enzyme having dehydrogenase activity,
providing a keto acid-CoA which will yield R-(-)-hydroxyacyl-CoA by the dehydrogenase, wherein said R-(-)-hydroxyacyl-CoA will serve as a substrate for polyhydroxyalkanoate synthase; and
producing said polyhydroxyalkanoate.

2. The method of claim 1 wherein the transgenic organism further comprises a DNA sequence encoding an enzyme having thioesterase activity; an enzyme having acyl-CoA synthetase activity; an enzyme having thiolase activity; and an enzyme having polyhydroxyalkanoate synthase activity.

3. The method of claim 2 wherein the enzyme having dehydrogenase activity is a yeast 3-hyroxyacyl-CoA dehydrogenase.

4. The method of claim 3 wherein the transgenic organism is a plant.

5. The method of claim 4 wherein the transgenic organism is a plant selected from the group consisting of: potato, sweet potato, cassava, beet, alfalfa, Arabidopsis and tobacco.

6. A cloning vector comprising foreign DNA encoding an enzyme having dehydrogenase activity, which will produce R-(-)-hydroxyacyl-CoA from a keto acid-CoA.

7. The cloning vector of claim 6 wherein the cloning vector further comprises a DNA sequence encoding an enzyme having thioesterase activity; an

enzyme having acyl-CoA synthetase activity; an enzyme having thiolase activity; and an enzyme having polyhydroxyalkanoate synthase activity.

8. The cloning vector of claim 7 wherein the enzyme having dehydrogenase activity is a yeast 3-hyroxyacyl-CoA.

9. A host cell comprising foreign DNA encoding an enzyme having dehydrogenase activity, which will produce R-(-)-hydroxyacyl-CoA from a keto acid-CoA.

10. The host cell of claim 9 wherein the host cell further comprises a DNA sequence encoding an enzyme having thioesterase activity; an enzyme having acyl-CoA synthetase activity; an enzyme having thiolase activity; and an enzyme having polyhydroxyalkanoate synthase activity.

11. The host cell of claim 10 wherein the enzyme having dehydrogenase activity is a yeast 3-hyroxyacyl-CoA.

12. A transgenic organism comprising foreign DNA encoding an enzyme having dehydrogenase activity, which will produce R-(-)-hydroxyacyl-CoA from a keto acid-CoA.

13. The transgenic organism of claim 12 wherein the transgenic organism further comprises a DNA sequence encoding an enzyme having thioesterase activity; an enzyme having acyl-CoA synthetase activity; an enzyme having thiolase activity; and an enzyme having polyhydroxyalkanoate synthase activity.

14. The transgenic organism of claim 13 wherein the enzyme having dehydrogenase activity is a yeast 3-hyroxyacyl-CoA.

15. The transgenic organism of claim 14 wherein the transgenic organism is a plant.

16. The transgenic organism of claim 15 wherein the transgenic organism is a plant selected from the group consisting of: potato, sweet potato, cassava, beet, alfalfa, Arabidopsis and tobacco.

17. A method for producing a polyhydroxyalkanoate in a host comprising:

selecting a host for expression of genes encoding enzymes required for synthesis of a polyhydroxyalkanoate;

introducing into said host structural genes encoding enzymes selected from the group consisting of: a thioesterase, an acyl-CoA synthetase, a thiolase, a hydroxyacyl-CoA dehydrogenase, and a polyhydroxyalkanoate synthase;

expressing the enzymes encoded by the genes; and

providing the appropriate substrates for the expressed enzymes to synthesis the polyhydroxyalkanoate.

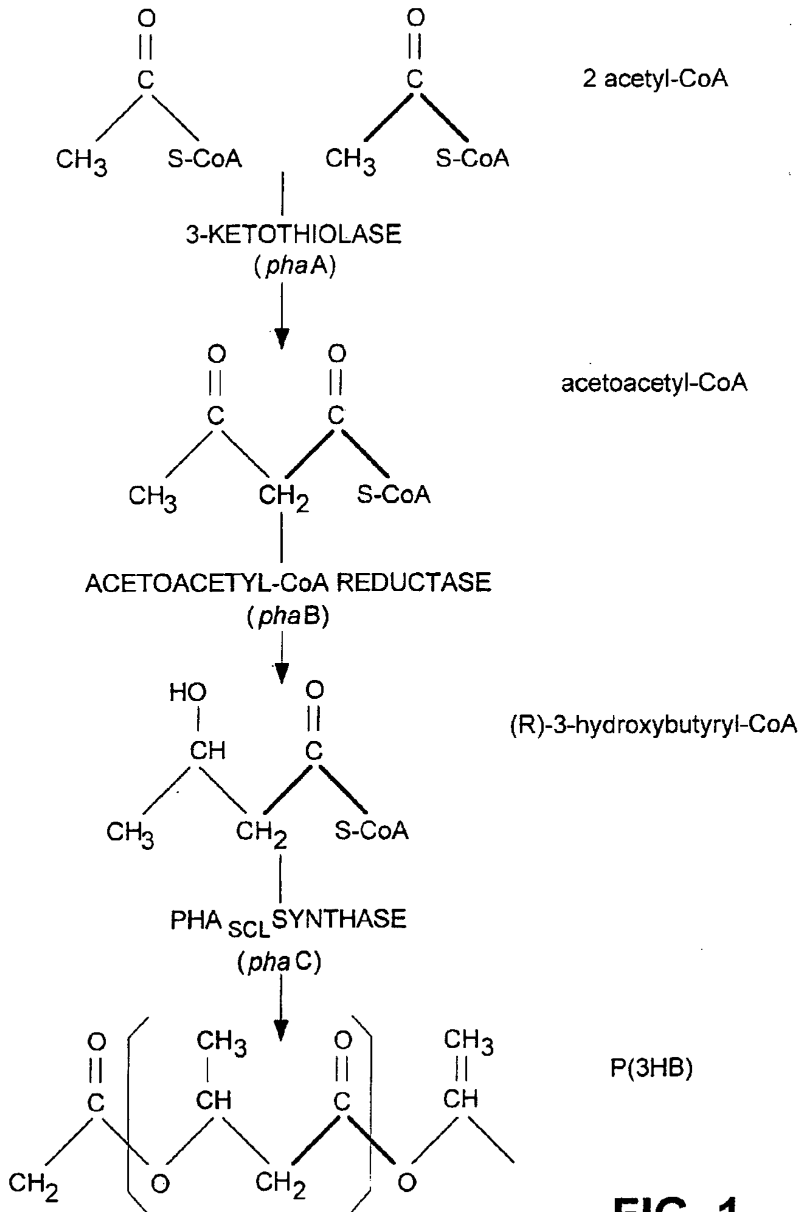

O
C
CH3
S-CoA
2 acetyl-CoA
3-KETOTHIOLASE
(phaA)
acetoacetyl-CoA
CH2
ACETOACETYL-CoA REDUCTASE
(phaB)
HO
CH
(R)-3-hydroxybutyryl-CoA
PHA SCL SYNTHASE
(phaC)
P(3HB)

**FIG. 1**

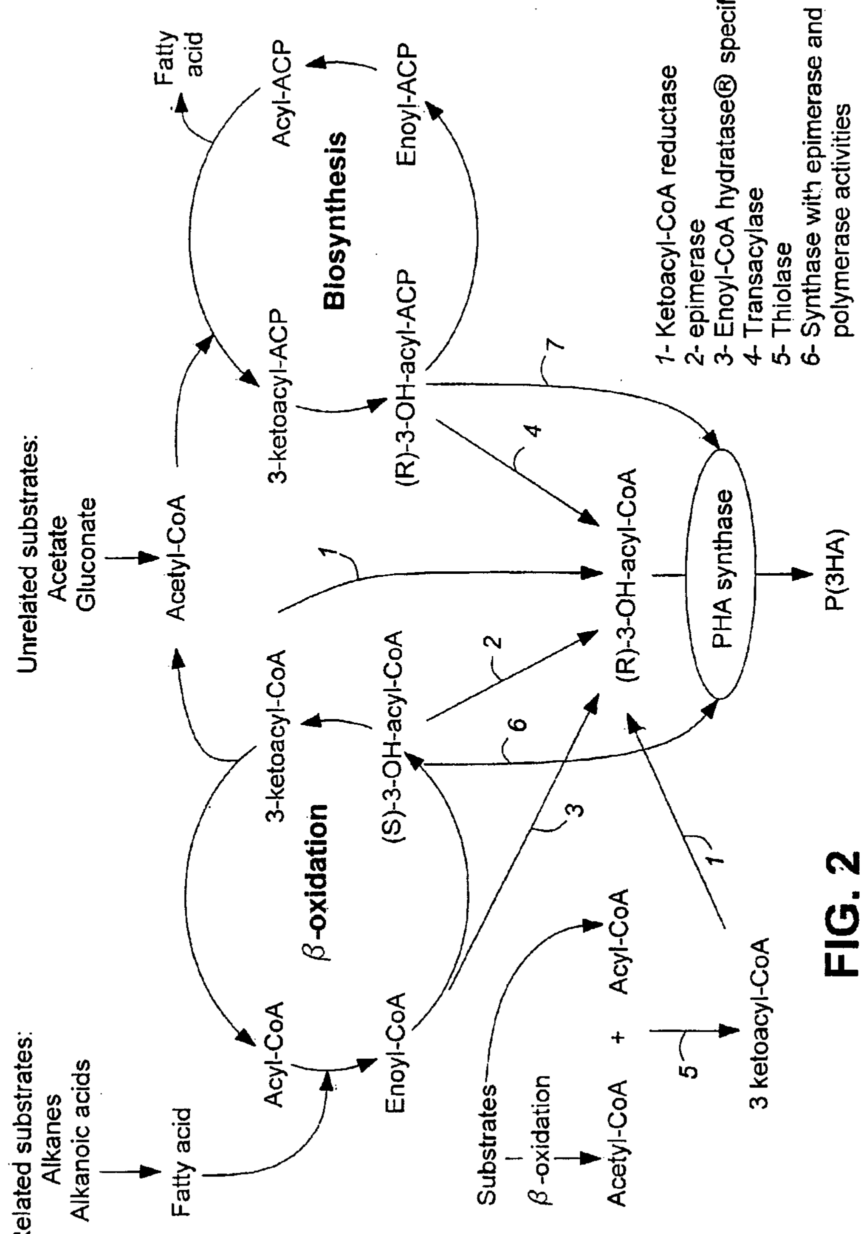

Related substrates:
Alkanes
Alkanoic acids
Fatty acid
Acyl-CoA
Enoyl-CoA
$\beta$-oxidation
3-ketoacyl-CoA
(S)-3-OH-acyl-CoA
Unrelated substrates:
Acetate
Gluconate
Acetyl-CoA
3-ketoacyl-ACP
(R)-3-OH-acyl-ACP
Biosynthesis
Acyl-ACP
Enoyl-ACP
Fatty acid
(R)-3-OH-acyl-CoA
PHA synthase
P(3HA)
Substrates
$\beta$-oxidation
Acetyl-CoA
Acyl-CoA
3 ketoacyl-CoA
1
2
3
4
5
6
7
1- Ketoacyl-CoA reductase
2- epimerase
3- Enoyl-CoA hydratase® specific
4- Transacylase
5- Thiolase
6- Synthase with epimerase and polymerase activities

FIG. 2

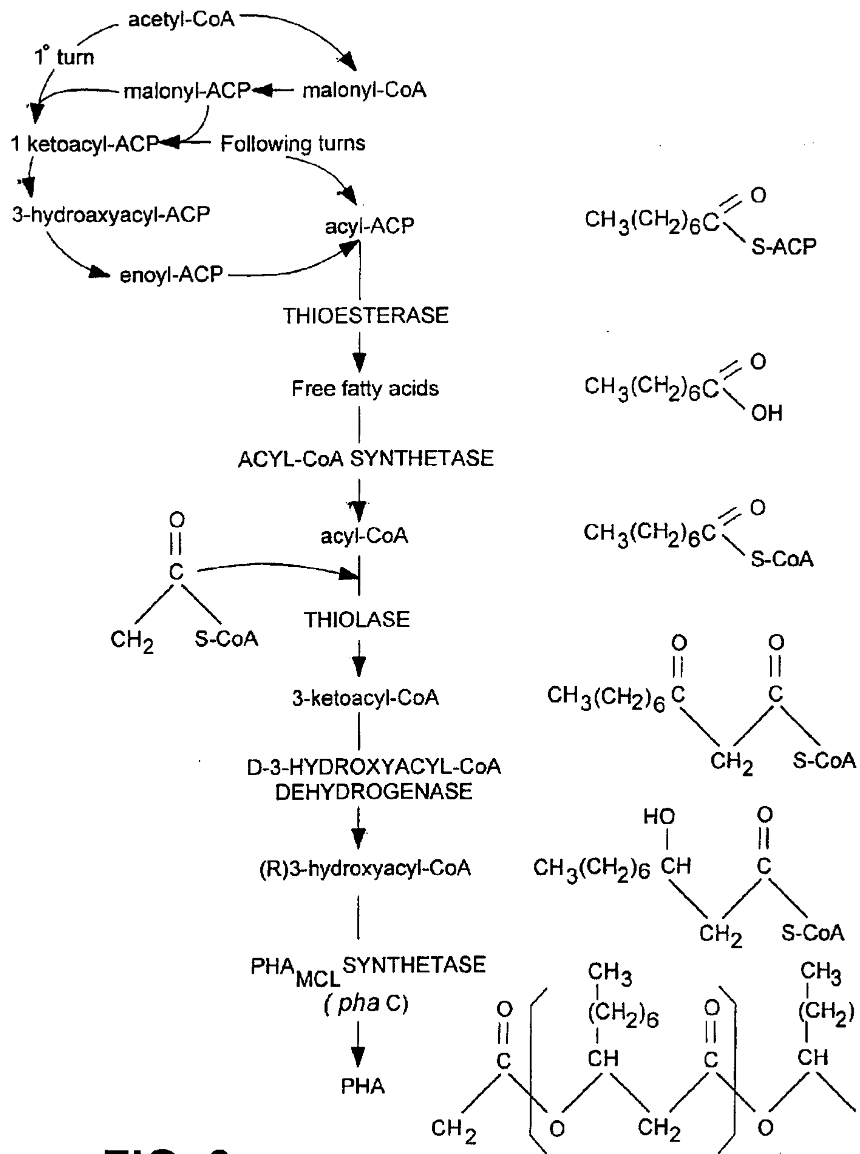
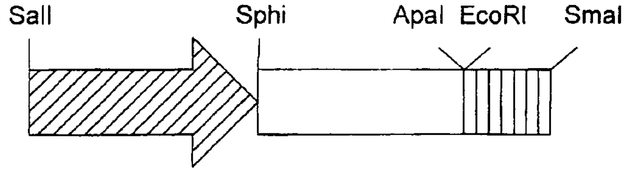
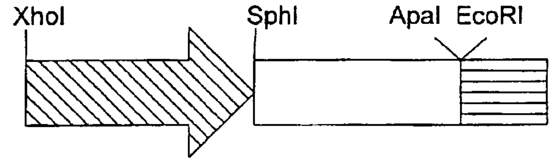

acetyl-CoA
1° turn
malonyl-ACP
malonyl-CoA
1 ketoacyl-ACP
Following turns
3-hydroaxyacyl-ACP
acyl-ACP
enoyl-ACP
THIOESTERASE
Free fatty acids
ACYL-CoA SYNTHETASE
acyl-CoA
THIOLASE
3-ketoacyl-CoA
D-3-HYDROXYACYL-CoA
DEHYDROGENASE
(R)3-hydroxyacyl-CoA
PHAMCL SYNTHETASE
( pha C)
PHA
S-ACP
OH
S-CoA

FIG. 3




RbscK-DH-3'nc in pKitmus and pCambia 2300




35S.C4PPDK.DH.3'nc in pUC18

ALFALFA RUBISCO PROMOTER

3' NON-CODING REGION OF ALFALFA RUBISCO

35S-C4PPDK PROMOTER

NOS

3-HYDROXYACYL-CoA DEHYDROGENASE

**FIG. 4**

GTTAACTACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATT
TTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCA
ATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTT
TTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGA
TGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAA
GATCCTTGAGAGTTTTCGCCCCGAAGAACGTTCTCCAATGATGAGCACTTTTAAAGTTCT
GCTATGTGGCGCGGTATTATCCCGTGTTGACGCCGGGCAAGAGCAACTCGGTCGCCGCAT
ACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGA
TGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGC
CAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACAT
GGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAA
CGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAAC
TGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAA
AGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATC
TGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCC
CTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAG
ACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTA
CTCATATATACTTTAGATTGATTTACCCCGGTTGATAATCAGAAAAGCCCCAAAAACAGG
AAGATTGTATAAGCAAATATTTAAATTGTAAACGTTAATATTTTGTTAAAATTCGCGTTA
AATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTAT
AAATCAAAAGAATAGCCCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCCA
CTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGATGGC
CCACTACGTGAACCATCACCCAAATCAAGTTTTTTGGGGTCGAGGTGCCGTAAAGCACTA
AATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCGAACGTGGCGA
GAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCA
CGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTAAAAGG
ATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCG
TTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTT
CTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTG
CCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATA
CCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCA
CCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAG
TCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGC
TGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGA
TACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGG
TATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAAC
GCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTG
TGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGG
TTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTAATGTGAGTTAGCTCACTCATTAGGC
ACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATA
ACAATTTCACACAGGAAACAGCTATGACCATGATTACGCCAAGC<u>GTCGAC</u>ctgcaggtca
acggatcaaatgattcaatatttggcttgatgaaattagagaaaatgaaaaattggattt
ctaagtttgattgttattttgagatagaaaaggaaaaatctctaatctcttacgcaagac
ctgcctcaaccacttgataaactcttttgtctacgtattgaaaacaaaagaggcaaataa
acatctagccaaatgaaacaccaataatgctttaaacaaaatggaataattgcatcatca
attaatctttataagtgagaattttccctcctataataatgcgctaggtatcaattttca
actctgaaatataaagcttcaagcgtgtgttatcaaaaatcaagcacagtaaaatcataa

FIGURE 5A

gcagaatcattggtgatgctaatagttgatgtggaatcgaacaatgttcatattctgata
ccttgttggtgacaagtcaagacccttatagacttgaattttgtctgagttgatgatttc
agaaggggaatctagtatctaagtagatggtaaatttatttttccaattccagttgctt
ccattatgaacaaaccttattcttttaggctaatattgaggaacaaaagccacggaatat
tttttttatgtataacctaagaaaaagacaataataaaaataattaaaaactacaacaga
tgattttggacttgaatcgaattggattaatcttatacatgttgtcgataaggatactag
ttatatgaagaagagaatcaattgaaactttatttgtgctatatataatgatttatgata
tatggagagagggatggcagaatatgcaagtttggaatcaattctggacattcatggagg
gcgggtttatcatcgtgggtgtggtaggggtggctgaggttctagggctacccagtactt
ttatgatgttgttgtagtattttgaacaaatttgtttttttaattttatgtttgaatattg
ggtttgtaacatatggataatttgtttttaattttatgttcgaaaattgtgttgtttttg
atcattttcattacaatatttatttatttattcacgaatgcatgtttatatcaacaaatt
atataatctgtatgtatcatagtgaaaacaaactctgtttttcttttgatacctttcaga
ttatataatttgaaatgtcataaaacagtttagattatataatctgaaatatgcgttttt
tacaccacattttgcattttgtgaggtgtttgacacttttcggattatataagtcaaatt
attttaagaagtttcggattatatatctgaaacatatgtttaactgacacatacacaaac
atctctagggtgatttgtcttccaatagtttttatactgtttggattatataatccgaat
caaggttaagaaaaaattagggcgctcgaaaaccaaatagggtgggaaaagtaatgacca
atattgattaccctataaggagccaaagcctgaaaaaagtaccatacatgattgatattt
gtggaggcattaatagtcacaaaactacacgtggcaattttatattggtggctaatgata
aggctagcacaaaaatttccattcctgtgtggttgatatggcagcaaagtttatcatatt
cacaaccaacaaaatggtattatgaagcattaccacaatttataagaccataatattgga
aataggaaaataaaaacattatatatagcaagtttgagtataagctttgcaattcaagca
gaagtacatcttactttactagtgaactaagtaagggagaaaaaaaatggcttcctctat
gatgtcctcttcagctgtgactacagttaaccgtgcctcttcggtacaatctggcgtggt
ggctccattcgttggactaaagtccatggctggcttcccagttacaaaggtcaacaaaga
cattacttccattgcaagcaatggtggaagagtaaactGCATGCtaatgcctggaaattt
atccttcaaagatagagttgttgtaatcacggccgctggaggggcttaggtaaggtgta
tgcactagcttacgcaagcagaggtgcaaaagtggtcgtcaatgatctaggtggcacttt
gggtggttcaggacataactccaaagctgcagacttagtggtggatgagataaagaaagc
cggaggtatagctgtggcaaattacgactctgttaatgaaaatggagagaaaataattga
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ggatgtctcatttgcaaagatgacagaacgtgagtttgcatctgtggtagatgttcattt
gacaggtggctataagctatcgcgtgccgcttggccttatatgcgctctcagaaatttgg
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ttcagcagctaaaatgggcttagttggtttggcggaaaccctcgcgaaggagggtgccaa
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gttaccaccacatatcttgaaacagttaggaccggaaaaaattgttcccttagtactcta
tttgacacacgaaagtacgaaagtgtcaaactccattttgaactcgctgctggattctt
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tactcctgaagcaattttaaataagtggaaggaaatcacagactatagggacaagccatt
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aaaattacctcccaatgaacaaggctcagtgaaaatcaagtcgctttgcaacaaagtcgt
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cggtgcgaaggtagttgtaaatgacatcaaggatcctttttcagttgttgaagaaataaa
taaactatatggtgaaggcacagccattccagattcccatgatgtggtcaccgaagcgcc
tctcattatccaaactgcaataagtaagtttcagagagtagacatcttggtcaataacgc
tggtattttgcgtgacaaatctttttttaaaaatgaaagatgaggaatggtttgctgtcct
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acaaaagtctggatttattatcaatactacttctacctcaggaatttatggtaattttgg
acaggccaattatgccgctgcaaaagccgccatttagggttcagtaaaactattgcact
ggaaggtgccaagagaggaattattgttaatgttatcgctcctcatgcagaaacggctat
gacaaagactatattctcggagaaggaattatcaaaccactttgatgcatctcaagtctc

FIGURE 5A Cont'd

cccacttgttgttttgttggcatctgaagaactacaaaaatattctggaagaagggttat
tggccaattattcgaagttggcggtggttggtgtgggcaaaccagatggcaaagaagttc
cggttatgtttctattaaagagactattgaaccggaagaaattaaagaaaattggaacca
catcactgatttcagtcgcaacactatcaacccgtaaGGGCCCctcgaggagctcGAATT
Catttgagaatttactatctgccattgaaggaacatttttttctctccatttgttctgtt
tgtaatttcctttttctttttaaaggaaatgtctccagtgtttttttcggtatttgctttcg
gattttgaaatgcaaatagatggataagagttaattaatgaaatgatacttttattcatt
ctcaaattagtttcattaatggatatataaagataaagtaataactgcgcctatgcttcc
tttgcattgaagcaactgaactttacctaattgaaatatctttacccttggcaactaaat
gagtgaatgatgaattggtcgtcgaaattgtgagcatttttgtcaaaatagtcctgaaat
atgctatttgagaaataaatctgaactgtacgcatcagtaCCCGGGTGAGTCGTATTACG
GACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATC
GCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATC
GCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCTTCGCTTGGTAATAAAGCC
CGCTTCGGCGGGCTTTTTTTT

FIGURE 5A Cont'd

TCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTA
TCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAG
AACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCG
TTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGG
TGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTG
CGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGA
AGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGC
TCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGT
AACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACT
GGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGG
CCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTT
ACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGT
GGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCT
TTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTG
GTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTT
AAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGT
GAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTC
GTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCG
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FIGURE 5B

TAAGCAGCTCTCGGCTCCCTCTCCCCTTGCTCCGTGGATCGGCATGCTAATGCCTGGAAA
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FIGURE 5B Cont'd

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FIGURE 5C

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FIGURE 5C Cont'd

CAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGA
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FIGURE 5C Cont'd

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ATGGGTTGCGATGGTCGTCTTGCCTGACCCGCCTTTCTGGTTAAGTACAGCGATAACCTT
CATGCGTTCCCCTTGCGTATTTGTTTATTTACTCATCGCATCATATACGCAGCGACCGCA
TGACGCAAGCTGTTTTACTCAAATACACATCACCTTTTTAGACGGCGGCGCTCGGTTTCT
TCAGCGGCCAAGCTGGCCGGCCAGGCCGCCAGCTTGGCATCAGACAAACCGGCCAGGATT
TCATGCAGCCGCACGGTTGAGACGTGCGCGGGCGGCTCGAACACGTACCCGGCCGCGATC
ATCTCCGCCTCGATCTCTTCGGTAATGAAAAACGGTTCGTCCTGGCCGTCCTGGTGCGGT
TTCATGCTTGTTCCTCTTGGCGTTCATTCTCGGCGGCCGCCAGGGCGTCGGCCTCGGTCA
ATGCGTCCTCACGGAAGGCACCGCGCCGCCTGGCCTCGGTGGGCGTCACTTCCTCGCTGC
GCTCAAGTGCGCGGTACAGGGTCGAGCGATGCACGCCAAGCAGTGCAGCCGCCTCTTTCA
CGGTGCGGCCTTCCTGGTCGATCAGCTCGCGGGCGTGCGCGATCTGTGCCGGGGTGAGGG
TAGGGCGGGGGCCAAACTTCACGCCTCGGGCCTTGGCGGCCTCGCGCCCGCTCCGGGTGC
GGTCGATGATTAGGGAACGCTCGAACTCGGCAATGCCGGCGAACACGGTCAACACCATGC
GGCCGGCCGGCGTGGTGGTGTCGGCCCACGGCTCTGCCAGGCTACGCAGGCCCGCGCCGG
CCTCCTGGATGCGCTCGGCAATGTCCAGTAGGTCGCGGGTGCTGCGGGCCAGGCGGTCTA
GCCTGGTCACTGTCACAACGTCGCCAGGGCGTAGGTGGTCAAGCATCCTGGCCAGCTCCG
GGCGGTCGCGCCTGGTGCCGGTGATCTTCTCGGAAAACAGCTTGGTGCAGCCGGCCGCGT

FIGURE 5C Cont'd

GCAGTTCGGCCCGTTGGTTGGTCAAGTCCTGGTCGTCGGTGCTGACGCGGGCATAGCCCA
GCAGGCCAGCGGCGGCGCTCTTGTTCATGGCGTAATGTCTCCGGTTCTAGTCGCAAGTAT
TCTACTTTATGCGACTAAAACACGCGACAAGAAAACGCCAGGAAAAGGGCAGGGCGGCAG
CCTGTCGCGTAACTTAGGACTTGTGCGACATGTCGTTTTCAGAAGACGGCTGCACTGAAC
GTCAGAAGCCGACTGCACTATAGCAGCGGAGGGGTTGGATCAAAGTACTTTGATCCCGAG
GGGAACCCTGTGGTTGGCATG

FIGURE 5C Cont'd

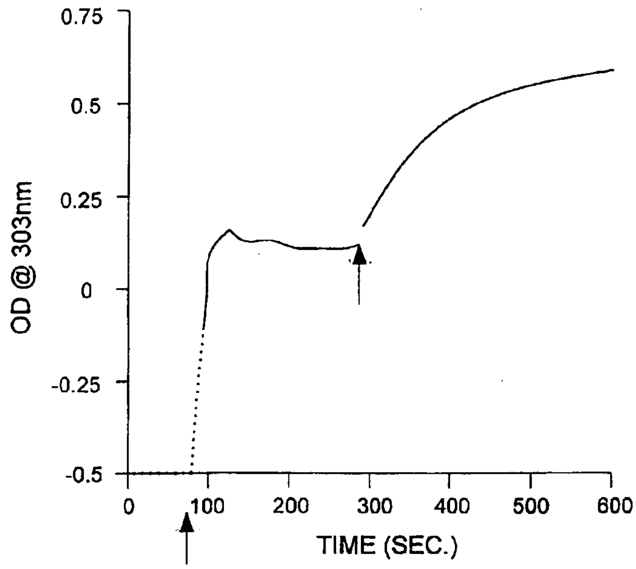
0.75
0.5
0.25
0
-0.25
-0.5
OD @ 303nm
0
100
200
300
400
500
600
TIME (SEC.)

**FIG. 6A**

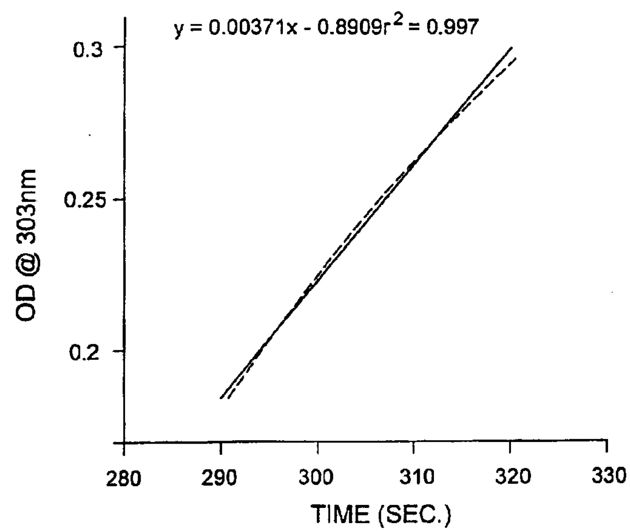
y = 0.00371x - 0.8909r² = 0.997
0.3
0.25
0.2
OD @ 303nm
280
290
300
310
320
330
TIME (SEC.)

**FIG. 6B**

INTERNATIONAL SEARCH REPORT

Inter    nal Application No
PCT/CA 00/00275

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/52 C12N15/53 C12N15/54 C12N15/55 C12N15/82
A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
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"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 20 July 2000 | 07/08/2000 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Kania, T |

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PCT/CA 00/00275

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